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THE NITROGEN METABOLISM OF PURE CULTURES OF RUMINAL BACTERIA[1/]

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The activities occurring in the rumen depend on the types of microorganisms selected by a particular ruminal environment and the effect of the particular environment on the potential activities of these microorganisms. Although a great deal of information on ruminal activities and factors affecting them can be obtained using various in vivo and in vitro techniques involving the mixed rumen microbial population or crude fractions of it, a complete understanding of the rumen fermentation rests on equating quantitatively and qualitatively the activities of the mixed flora and fauna with the activities and ecology of the individual species. At the present time the main methods available for obtaining information on individual species involve the judicious use of pure cultures.

The purpose of my discussion today is to present some of the more recent information on nitrogen metabolism by pure cultures of ruminal bacteria. For a more complete discussion of nitrogen metabolism in the rumen with information on microbial species involved the paper by Dr. P. N. Hobson (1) is recommended.

Protein catabolism. It is now generally believed that breakdown of protein of food passing into the rumen is quite extensive (40-90% being hydrolyzed) and that the general mechanism is similar to that occurring in many other systems, i. e. hydrolysis of the protein to amino acids and peptides and then breakdown of these smaller units to form ammonia, carbon dioxide, volatile fatty acids and, probably, some methane. It is evident that both bacteria and ciliate protozoa are involved in protein breakdown but very little is as yet published concerning the individual species that bring about these reactions.

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Appleby (2), Hunt and Moore (3) and Blackburn and Hobson (4) isolated proteolytic facultative anaerobes but the latter workers believed that these accounted for only a fraction of the ruminal proteolytic activity. Fulgum et al. (5) and our group (6) found nonsporeforming anaerobes present in large numbers in the rumen to be active in gelatin and/or casein digestion and more recent results of Blackburn and Hobson (41) have confirmed this.

The main species that appear to be involved in proteolysis based on work at Beltsville, using nonselective cultural methods, appear to be Bacteroides ruminicola and, probably to a less extent, some Butyrivibrio strains and Eubacterium sp. (7). However, work as yet unpublished at Virginia Polytechnic Institute and the Rowett Research Institute, using selective cultural procedures, will undoubtedly show further proteolytic species.

The nature of the proteases produced by the pure cultures have not yet received attention. It is of interest that the more numerous anaerobic proteolytic species do not appear to be strongly proteolytic when compared to organisms such as Clostridium sporogenes, a proteolytic species common in poor quality silage, and they appear to require a carbohydrate energy source for growth. It seems possible that endocellular proteases, liberated by cell lysis, are of as much or more importance in the breakdown of protein in the rumen as extracellular systems secreted by microorganisms during growth. Many ruminal bacteria are known to lyse quite rapidly.

Warner (8) and, more recently, others have shown that the potential proteolytic activity of the mixed ruminal flora varies to a relatively small degree in animals fed varying amounts of protein. The pure culture studies of Bryant and Burkey (6) tended to support this finding in that only small variations in numbers of gelatin liquefying bacteria were found in the rumens of cattle fed widely differing rations.

Amino Acid Catabolism. Shazly (9) showed that ammonia and volatile fatty acids are produced from amino acids by rumen liquor and washed mixtures of ruminal microbes and that the deaminative enzymes are adaptive in organisms already present or a deaminative flora is induced by amino acids. Since this time his findings have been confirmed and it has been shown more or less conclusively that most amino acids are broken down by the mixed ruminal population. However, work has only just begun in determining what species of microorganisms attack amino acids and what mechanisms are involved.

Results on NH_3 production from casein hydrolysate by predominant ruminal bacteria indicated that under the usual conditions Bacteroides ruminicola would be the most important ammonia-producing bacterium in mature cattle with Butyrivibrio and Selenomonas contributing (10). P. elsdenii would be active in mature cattle fed certain high grain rations and in calves.

Data shown in Table 1 indicate that the week-old calf receiving only milk maintains a high proportion of ammonia-producing bacteria in the rumen and that the bacterial species differ from those present in older calves (6 weeks) or mature ruminants.

Table 1. The occurence in calves of groups of ruminal bacteria that produce ammonia from casein hydrolysate[a]

	Percent of Total Isolates					
Bacterial Group	1 wk.	3 wk.	6 wk.	9 wk.	13 wk.	Adult
Peptostreptococcus sp. (C1)	15.3					
P. elsdenii (C2)		10.0	2.0			
Bacteroides sp. (R1)	25.0	6.7				
Bacteroides sp. (R2)	15.3					
Fusobacterium sp. (R3)	8.3	5.0				
Bacteroides ruminicola			8.2	1.6	22.2	16.0
Butyrivibrio sp.[b]			2.0	6.6	2.8	4.9
Selenomonas ruminantium			6.1	1.6		5.0
Total NH_3 producers (%)	63.9	21.7	18.3	9.8	25.0	29.7
Total Strains (number)	72	60	49	61	36	81

[a]Data modified from Bryant *et al.* (*J. Diary Sci.*, *41*, 1947) and Bladen *et al.* (Appl. Microbiol., *9*, in press)

[b]Estimated on basis of above reference that only 10% of butyrivibrios produce ammonia.

Results shown in Table 2 obtained from presumptively identified freshly isolated strains show that a cow on a high protein ration did not support any more culturable ammonia-producing bacteria than an animal on a low protein ration. This suggests that the adaptation described by Shazly in which bacteria from animals fed a higher protein ration produced more ammonia was due to production of more enzyme by the same species of bacteria rather than a change in bacterial species. However, the present results should be checked using rations containing a more soluble protein such as casein and using a more appropriate experimental design; and the ammonia-producing potential of the mixed flora should be concurrently measured.

Table 2. Occurrence in cattle fed rations containing low, medium and high levels of protein of presumptively identified groups of ruminal bacteria that produce ammonia (1μM or more per ml) from casein hydrolysate[a]

Presumptive Identification[b]	Percent of Strains			
	6% C.P.[c]	15% C.P.	22% C.P.	Total
Bacteroides ruminicola	18.6	9.3	12.5	13.7
Selenomonas	6.2	0	3.4	2.6
Butyrivibrio	0	2.3	5.7	2.6
Unknown	9.3	8.1	6.8	8.1
Others	1.0		1.1	0.7
Total ammonia producers	35.1	19.8	27.3	27.7
Total isolates (No.)	97	86	88	271

[a]Modified from Bladen et al. (10)

[b]Non-ammonia producers included Ruminococcus sp., Bacteroides succinogenes, Lachnospira, Eubacterium ruminantium, Streptococcus sp., Succinivibrio, Borrelia and Bacteroides amylophilus.

[c]Rations were timothy hay, alfalfa hay plus concentrates and the latter plus extra soybean oil meal. Culture counts on the three rations were not significantly different.

In evaluating the importance of a ruminal bacterium in catabolism of amino acids, it is advantageous to know which amino acids are catabolized, the rate of the reaction and what products are produced in addition to the numbers present in the rumen. Lewis et al. (11) established that P. elsdenii produced NH_3, H_2, CO_2 and certain volatile fatty acids from threonine, serine and cysteine. No other ruminal organisms have been studied in such detail. B. amylogenes (12) produced sulfide from cysteine as was also found to be true of Selenomonas (13).

Bladen et al. (14) spent considerable time attempting to determine which amino acids were catabolized by a strain of B. ruminicola. Considerable variation occurred in results on ammonia production from single amino acids in growth media or from single or pairs of amino acids incubated with washed suspensions. However, it was apparent that the organism produced ammonia from both D-and L-serine, L-cysteine, and L-aspartic acid.

During attempts to obtain more consistent results on ammonia production from amino acids using various modifications of the washed cell technique it was found that casein hydrolysate gave much more consistent results than single or pairs of amino acids regardless of the modification in the technique.

It was believed that perhaps with more sensitive methods the breakdown of other amino acids could be detected. In subsequent studies (14) it was shown by microbiological assay that one or more of the branched-chain volatile acids required by certain ruminal cellulolytic bacteria was produced by the washed cell suspensions incubated with casein hydrolysate and studies with $2C^{14}$-leucine plus casein hydrolysate indicated that isovaleric acid was produced.

Further studies will be necessary to adequately establish the significance of B. ruminicola in amino acid breakdown in the rumen, but the results to date indicate that it is of some improtance in this respect.

It is known that certain purines are attacked by mixed ruminal bacteria with production of acetic acid, CO_2 and NH_3 but the organisms responsible are not known (15).

Nitrate is reduced by strains representing numerous species but reduction products other than nitrite have not been determined.

The Nitrogen Requirements for Growth of Ruminal Bacteria.

It has become increasingly evident that the nitrogen requirements other than nitrogen in B-vitamins for growth of ruminal bacteria are usually very simple. Many strains of S. bovis can be grown with ammonia (16,17), arginine (16,18), glutamine (16,17), asparagine (17), citrulline,

serine, histidine or glucosamine (16), or glutamic acid plus arginine (17,18) as the sole source of nitrogen. L. bifidus strains may grow with ammonia (19), ammonia plus a small amount of methionine (20), or ammonia plus cysteine (21) as the only sources of nitrogen. Ammonia also serves as the sole source of nitrogen for Lachnospira (22) and the cellulolytic anaerobes, B. succinogenes and Ruminococcus (most strains, 23). A strain of Butyrivibrio did not require any single amino acid (24).

A few strains of ruminal bacteria appear to have somewhat more complex nitrogen requirements. Two strains of Ruminococcus (25,26) and a strain of B. ruminicola (27) required factors (presumably amino acids) in acid hydrolysed casein and one ruminococcus required adenine and guanine (26). Some strains of L. bifidus required purines and/or pyrimidines (19).

Factors believed to be peptides (also possibly amides) have been shown to be highly stimulatory but not required by several strains of ruminal bacteria (27,28).

Not only do many of these bacteria have very simple nitrogen requirements but it is now evident that ammonia nitrogen is sometimes preferred or is essential for synthesis of most cellular nitrogen compounds. Organisms shown to preferentially assimilate large amounts of ammonia when presented with both ammonia and a large amount of amino acid nitrogen include strains of Butyrivibrio (24) and L. bifidus (21). Strains of the cellulolytic anaerobes of the genus Ruminococcus and Bacteroides succinogenes not only prefer ammonia but amounts approximating the amount of cell nitrogen produced is essential (23). With the latter organism, the nitrogen of glutamine and asparagine is also utilized but at much slower rates than ammonia.

Although urea is utilized directly by some microorganisms no rumen microorganisms have as yet been found that have this capacity. It is probable that most urea nitrogen is first hydrolyzed to ammonia and then used. Although some urease-producing microorganisms have been isolated, it is not at all certain whether they account for a significant amount of the urease of rumen fluid (20).

The use of carbon compounds in synthesis of rumen bacterial cell nitrogen compounds.

Only a small amount of work has been done to determine the sources of carbon used by ruminal microorganisms in the synthesis of cellular nitrogen compounds. It is apparent that mixed ruminal bacteria and many pure cultures can use carbon of the carbohydrate energy source for much of the carbon used in protein and nucleic acid synthesis.

Available information suggests that a considerable amount of carbon dioxide and/or bicarbonate is utilized in synthesis of cellular nitrogen compounds even when exogenous organic nitrogen is available. It was shown to be highly stimulatory or required for growth of strains of S. bovis (17,29), L. bifidus (20), and Butyrivibrio (24) when grown in chemically defined media, especially when few organic nitrogen compounds were supplied. Another strain of S. bovis was shown to synthesize most of its cellular aspartic acid and some threonine, glutamic acid and nucleic acids via carbon dioxide fixation reactions even in the presence of exogenous amino acids, purines and pyrimidines (30). Similar results were obtained with ruminal lactobacilli (31).

Many species of ruminal succinic acid-producing bacteria require high levels of CO_2 for growth (7). A non-ruminal organism having similar fermentation products was shown to require CO_2 for fermentation of carbohydrate and CO_2 was incorporated into succinate via phosphoenol-pyruvate (32). It was suggested that the succinic acid-producing ruminal bacteria brought about a similar reaction and, if so, it is likely that CO_2 would be used in synthesis of aspartic acid and related nitrogen compounds, particularly if exogenous amino acids were not present or appear to be utilized to a very limited extent as in the case of ruminococci and B. succinogenes (23,33).

Certain observations suggest that volatile fatty acids may be important precursors of protein carbon in ruminal microorganisms. Allison et al. (33) showed that isovaleric acid (both 1 and 3 C^{14}) was incorporated into cell leucine and lipid of a strain of R. flavefaciens even when high levels of exogenous leucine were available. Wegner (34) showed that the same strain incorporated isobutyrate into valine. However, another strain of ruminococcus incorporated isobutyrate, but not isovalerate into cell lipid but not into protein (33). It would be of interest to determine the extent to which the various ruminal volatile acids are incorporated into protein of the mixed ruminal flora.

The few ruminal bacteria so far studied appear to have a high capacity for synthesis of cellular organic sulfur compounds. S. bovis (18) and Bacteroides succinogenes (35) will grow with sulfide as the sole source. Lachnospira utilizes sulfate but appears to prefer organic sulfur compounds (22). A strain of Ruminococcus flavefaciens was stimulated by sulfide even when cysteine and methionine was present in the medium (36). It is suggested that this strain may be stimulated by sulfide because of its very limited ability to utilize exogenous amino acids including methionine and cysteine (23,33) and one may speculate that sulfide is actually essential for its growth. A strain of L. bifidus required methionine or homocysteine (20).

Some nutrient requirements of freshly isolated predominant bacterial strains.

Our increased knowledge of the nutritional requirements of ruminal bacteria has made it possible to culture most of them in defined or in semi-defined media in which enzymatic hydrolysate of casein is the only unknown constituent. Table 3 shows some media that were used to screen freshly isolated predominant strains for a few nutritional requirements (37). Samples of ruminal contents were collected by stomach tube from a 1300 lb. cow fed alfalfa hay (11.3 lb.) and grain mixture (7.5 lb.) at 7:00 and water at 5-7:00 and 13-14:00 daily. Samples from which isolations were made were collected 6 hr. (the earliest time after feeding at which near maximal colony counts were obtained) and 22 hr. after feeding. Colony counts were as follows (average of 5 roll tubes of 40% clarified rumen fluid, glucose, cellobiose, starch agar incubated 3 days): 2.2, 2.0, 0.63, 2.9, 5.2 and 3.5 billion per gram at 5,7,9, 11, 13 and 15:00.

Table 3. Media used to screen freshly isolated ruminal bacteria for amino acid and/or peptide, ammonia, volatile fatty acid, hemin and other growth factor requirements

Ingredients	Medium[a]							
	A	B	C	D	E	G	H	I
$(NH_4)_2 SO_4$ (0.09%)	+	+	+	+		+	+	+
Volatile fatty acids[b]		+		+	+			+[e]
Casein hydrolysate[c]			+	+	+	+	+	+
Vitamin mix[d]	+	+	+	+	+			+
Hemin (2µg/ml.)	+	+	+	+	+	+		+
Clarified rumen fluid (20%)						+	+	

[a] All media contained 0.3% glucose (cellobiose for ruminococci and maltose for B. amylophilus), 0.0001% resazurin, 0.4%, Na_2CO_3, 0.025% each of cysteine. $HCl \cdot H_2O$ and $Na_2S \cdot 9H_2O$, 0.09% each of KH_2PO_4 and NaCl, 0.002% each of $CaCl_2$ and $MgCl_2 \cdot 6H_2O$, 00.01% $MnCl_2 \cdot 4H_2O$, 0.001% $CoCl_2 \cdot 6H_2O$ and was equilibrated with 100% CO_2 gas.

[b] 0.306% sodium acetate$\cdot 3H_2O$, 0.0017% sodium isobutyrate and 0.0019% each of sodium valerate, isovalerate and DL-a-methyl-n-butyrate.

[c] 0.2% enzymatic hydrolysate (vitamin-free) aerated at pH 12 (NaOH) to remove ammonia and neutralized with H_2SO_4.

[d] Two ug./ml. each of thiamin HCl, Ca-D-pantothenate, nicotinamide, riboflavin and pyridoxal; 0.1µg./ml. p-aminobenzoic acid, 0.05µg./ml each of biotin, folic acid and thioctic acid, and 0.02 µg./ml. of vitamin B_{12}.

[e] Acetate only.

It is evident (Table 4) that most strains of culturable bacteria (86%) grew at least fairly well in media with enzymatic hydrolysate of casein as the only ingredient not defined. This means that few if any of the strains required nucleic acids or their more complex decomposition products, co-enzymes, long chain unsaturated fatty acids or many other

Table 4. Some nutritional characteristics of freshly isolated strains of predominant ruminal bacteria based on the growth of strains in media with and without enzymatic hydrolysate of casein (A.A.), Ammonia, volatile fatty acids (V.F.A.), and hemin.

Nutritional Characteristic [a]	% of Total Strains		
	6 hr.	22 hr.	6+22 hr.
1. A.A. essential	7.1	4.5	5.8
2. A.A. essential in absence of V.F.A.	28.6	20.4	24.4
3. Utilize either NH_3 or A.A.	38.1	22.7	30.2
4. a. NH_3 essential, V.F.A. not essential	9.5	2.3	5.8
b. NH_3 and V.F.A. essential	11.9	27.3	19.8
5. Little or no growth	4.8	22.6	14.0
6. Hemin essential	40.5	22.7	31.4
Total strains studied	42	44	86

[a]Groups 1 through 5 each represent different strains. Group 6 includes strains also in groups 1, 2 or 3. Presumptively identified strains were found in the groups as follows (6 and 22 hr. samples): Bacteroides succinogenes, group 4b (2,5); B. ruminicola subsp. ruminicola (all in group 6), group 3(4,1), group 2 (10,7), group 1 (3,2); B. ruminicola subsp. brevis, group 3 (0,2), group 2 (2,2); B. amylophilus, group 4a (4,1); Eubacterium ruminantium, group 4b (1,3); unknown gram variable coccus to rod, group 4b (0,3); Ruminococcus, group 4b (1,0); Lachnospira, group 3 (5,3); Selenomonas, group 3 (0,1); Borrelia, group 5 (1,0); Other gram negative curved rods mainly Butyrivibrio, group 3 (10,3), group 4b (1,1), group 5 (0,9); gram negative motile lancets, group 5 (1,0).

growth factors required by some microorganisms. Other studies indicate that practically all strains would require one or more B-vitamins. None of the 14% of strains that grew poorly in the complete medium D grew better with menadione added.

It was recently shown (37) that hemin would replace the rumen fluid requirement of Bacteroides ruminicola subsp. ruminicola and data in Table 4 show that this organism makes up a significant proportion of ruminal bacteria. Although this species is almost invariably among the predominant species in ruminal contents of animals fed a wide variety of rations it is often not present in as high proportions as found in the present animal.

The nitrogen requirements appear to be very simple in that 80% of the strains could be grown with ammonia as the main source of nitrogen other than cysteine which was present in all media. The strains that required amino acids all belonged in the B. ruminicola group, most strains of which do not require amino acids. This suggests that even the amino acid requiring strains would require few amino acids. In support of this, a preliminary study of one of these strains indicates that, given a choice between enzymatic hydrolysate of casein (43μMN per ml. medium) and ammonia (5μM per ml.), over half of the trichloroacetic precipitable nitrogen produced during growth was accounted for by ammonia uptake and only a small amount of UL-C^{14} amino acids was taken up (about 18% of the C^{14} taken up by S. bovis).

While 55% of the strains appeared to be highly adaptable in that they grew with either ammonia or amino acids as the main nitrogen source, many (26%) were more rigid in that ammonia was essential for growth.

The facts that most ammonia requiring strains also require volatile fatty acids (group 4b), that many strains required volatile fatty acids for growth with ammonia as the main source of nitrogen but not when amino acids were present (group 2) and that many strains that utilized either amino acids or ammonia for growth (group 3) were highly stimulated by volatile fatty acids, especially when amino acids were absent, suggests, as indicated above, a close relationship between amino acids and volatile fatty acids in the nutrition of ruminal organisms.

Strains stimulated or requiring volatile fatty acids in the presence of amino acids were not affected by acetate alone. This indicates that the longer chained acids were involved.

Although more predominant strains from other animals held under similar conditions should be studied before definite conclusions can be drawn, the results in Table 4 suggest that, while qualitatively the strains isolated from the 6-hr. and 22-hr. samples were similar, there appeared to be some quantitative differences.

More group 4a (B. amylophilus-like) strains and group 6 (B. ruminicola subsp. ruminicola-like) strains, organisms believed to be very important in starch digestion, were found in the 6-hr. sample; and more group 4b, mainly species that attack cellulose and/or pentosans, were found in the 22-hr. sample. It seems logical that organisms attacking the more rapidly metabolized grain constituents of the ration such as starch would grow more rapidly than those attacking fiberous constituents. However, this tendency would not be expected to be clear-cut because some species, e.g. B. ruminicola and butyrivibrios, attack both fiberous and more readily digestible constituents of the ration.

More poor growing, supposedly nutritionally exacting, strains (mostly Butyrivibrio-like) were present in the 22-hr. sample. This suggests that the present 6-hr. sample is similar to results of one-time-sampling of grain-fed animals and the 22-hr. sample is similar to results of one-time sampling of hay-fed animals. Several workers have isolated more rapidly and easily grown strains from animals fed mainly grain.

That the total of organisms that utilize amino acids and which have no ammonia requirement (groups 1,2 and 3) were more numerous in the 6-hr. sample (74%) than in the 22-hr. sample (48%) seems to fit with the observations of many workers that free amino acids and peptides are mainly present in the rumen for only a short period after feeding.

Summary and Conclusions

To obtain a complete understanding of the ruminal fermentation, the activities and ecology of the many individual species of microorganisms present in the rumen must be equated with the activities of the mixed flora and fauna. Pure culture studies are the main source presently available for information on the individual species.

A considerable amount of information is now available or will be available in the near future on thenumbers and species of bacteria responsible for proteolysis and amino acid catabolism in the rumen. However, a great deal of work remains to be done concerning the mechanisms involved in the individual species.

Little is known of the organisms attacking nucleic acids, those that produce ammonia from nitrate or intermediates in the reduction of nitrate, or those responsible for urea hydrolysis.

Nitrogen requirements for growth of many species of ruminal bacteria of diverse function are very simple in that ammonia often will serve as the main if not the only source of cell nitrogen other than that present in B-vitamins. Ammonia in amounts approximating the amount of cell

nitrogen produced is essential for growth of some of these species and some of the latter have been shown to have a very limited ability to utilize organic nitrogen compounds. Many ruminal bacteria are quite versatile in that they can utilize either ammonia or amino acids and/or peptides as the main source of nitrogen.

In addition to carbohydrate carbon, carbon dioxide and, perhaps, volatile fatty acids are important sources of carbon for protein synthesis by ruminal bacteria.

The few species so far studied suggest that ruminal bacteria have a high capacity for synthesis of cellular organic sulfur compounds.

Recent results indicate that hemin is an important growth factor for ruminal bacteria.

The Numbers and Species of Ruminal Bacteria that have been Grown in Pure Culture as Compared to the Total Ruminal Population

Dr. Pfander asked me to conclude my talk with a discussion of the numbers and species of ruminal bacteria that have been grown in pure culture as compared with the total present. Hungate (38) has recently discussed this and I will include some of his reasoning in my discussion.

One of the main reasons for believing that many important ruminal bacteria have not been cultured is the apparent discrepancy between culture counts and direct microscopic counts. However, really good comparisons of culture counts and direct microscopic counts have not been made. Gall et al. (39) perfected an accurate direct microscopic count and cultural counts sometimes approached the direct count. However, errors involved in the liquid culture count used were commonly 10 to 100 fold. Others (e.g. 6,40) used much more accurate culture counting methods (colony counts) and found that, in animals on mainly hay rations, culture counts accounted for only about 3 to 12% of the direct microscopic counts. Colony counts from animals fed mainly concentrate rations were higher and more closely approached the microscopic count.

In analyzing these data, especially those from forage-fed animals, the following points should be emphasized. (1) It is probable that some of the predominant bacteria in the rumen are not viable and it is also probable that the % viability would vary with the ration, kind of sample, and time of sampling. (2) Future work might show that all predominant species of bacteria cannot be grown on one medium. (3) In studies reported in the literature, colony counts were usually made after only 3 days of incubation. It has been known since the early studies that higher counts could be obtained by longer incubation times. We recently found that colony counts at 3 days were 67% (50-80) of 7-day counts and the latter, 80% of 2-week counts. (4) Better cultural procedures are now available. We recently modified the rumen fluid agar medium and obtain double the colony counts obtained with the old medium.

If comparable microscopic and colony counts of ruminal contents from forage-fed animals were made with the best methods now available, it is possible that the colony counts would be 10 to 30% of the microscopic count rather than 3 to 12%. Thus, some of the apparent difference between colony counts and microscopic counts tend to disappear.

A second reason for believing that many important ruminal bacteria have not been isolated is the fact that organisms present in numbers sufficient to bring about certain reactions known to occur in the rumen have not been found. Succinoxidase- and urease-producing organisms are possible examples of as yet unknown organisms. However, it should be emphasized that far from all organisms that have been isolated have been tested for these or many other types of reactions.

Reasons for believing that most kinds of predominant ruminal bacteria have been isolated include the following: (1) The gram reactions and morphology of predominant organisms in stained smears of ruminal contents are consistent with the morphology and gram reaction of the predominant bacteria cultured using the more successful cultural procedures. (2) Many distinctive morphological types identifiable in contents have been grown in pure culture, e.g. spirochetes, Selenomonas, P. elsdenii, S. bakeri, cellulolytic cocci, rosette-producing rods (B. succinogenes) (see 7 for references). (3) Practically all colonies in initial culture can be maintained in pure culture. (4) Many major metabolic processes known to occur in the rumen have been demonstrated in pure cultures of species present in large numbers in the rumen. (5) As people continue to isolate bacteria bringing about certain reactions, they are finding more and more that these bacteria belong to or are very closely related to groups already described, e.g. saponin and fructosan fermentation by B. ruminicola and Butyrivibrio (7), amino acid catabolism by B. ruminicola (10), glycerol fermentation by Selenomonas, proteolysis by B. amylophilus (41), volatile fatty acid requirement of Borrelia (42). (6) Our group, and presumably others, have isolated species on which data are as yet unpublished. (7) The nutrition of pure cultures seems to fit nicely with facts known concerning the ruminal environment and in some cases with mixed culture data. e.g. B_6, biotin, PABA, calcium and straight- and branch-chained volatile fatty acid requirements of the cellulolytic bacteria; sodium, ammonia and CO_2 requirement of some; energy sources and the lack of extensive requirements for organic nitrogen. (8) In our recent work where it was possible to double colony counts previously obtained, we have cultured the same organisms previously isolated. This suggests that much of the discrepancy between microscopic and cultural counts could be due to non-viability of some cells of species cultured by that method.

The above considerations leave me with the feeling that a majority of bacterial groups present in the rumen have been cultured. However, because of the great variation between strains in various groups which often makes it difficult to delineate species, it seems probable that many new varieties, subspecies or species of known genera, subgenera or species will be isolated in the future; and these may vary from the presently known strains in physiological characteristics of significance in the rumen but of minor significance in delineating the microbial species. Also, it is probable that some predominant groups have not been cultured, and even more probable that some groups of significance in the rumen but not present in numbers sufficient for isolation by non-selective methods have not been cultured.

Only continued studies by workers in different laboratories and using different approaches will give us the final answer to this question.

REFERENCES

1. Hobson, P.N. 1959, TID-7578, U.S. Government Printing Office, Wash. 25, D.C., p. 203-212.
2. Appleby, J.C. 1955, J. Gen. Microbiol., 12, 526-533.
3. Hunt, W.G. and Moore, R.O. 1958, Appl. Microbiol., 6, 36-39.
4. Blackburn, T.H. and Hobson, P.N. 1960, J. Gen. Microbiol., 22, 282-289.
5. Fulgum et al. 1958, Bacteriol. Proc., p. 22-23, and personal communication.
6. Bryant, M.P. and Burkey, L.A. 1953, J. Dairy Sci., 36, 218-224.
7. Bryant, M.P. 1959, Bacteriol. Revs., 23, 125-153.
8. Warner, A.C.I. 1956, J. Gen. Microbiol., 14, 749-762.
9. Shazly, K. el- 1952, Biochem. J. 51, 647-653.
10. Bladen, H.A. et al. Appl. Microbiol., 9, in press.
11. Lewis, D. et al. 1952, Second International Congress of Biochemistry, p.89.
12. Doetsch, R.N. et al. 1957, J. Gen. Microbiol., 16, 156-168.
13. Bryant, M.P. 1956, J. Bacteriol., 72, 162-167.
14. Bladen, H.A. et al. 1961, J. Dairy Sci., 44, 173-174 and unpublished data.
15. Jurtshuk, P. Jr. et al. 1958, J. Dairy Sci., 41, 190-202.
16. Prescott, J.M. et al. 1959, Proc. Soc. Exptl. Biol. Med., 102, 490-492.
17. Wolin, M.J. et al. 1959, J. Bacteriol., 78, 147.
18. Niven, C.F. et al. 1948, J. Bacteriol., 55, 601-606.
19. Wasserman, R.H. et al. 1953, J. Animal Sci., 12, 935-936.
20. Gibbons, R.J. and Doetsch, R.N. 1959, J. Bacteriol., 77, 417-428.
21. Phillipson, A.T. et al. 1959, Nature, 183, 402-404.
22. Emery, R.S. et al. 1957, Appl. Microbiol., 5, 363-366.
23. Bryant, M.P. and Robinson, I.M. 1961, Appl. Microbiol. 9, in press.
24. Gill, J.W. and King, K. W. 1958, J. Bacteriol., 75, 666-673.
25. Fletcher, D.W. 1956. Ph.D. Thesis, State College of Washington.
26. Ayers, W.A. 1958, J. Bacteriol, 76, 15-23.
27. Bryant, M.P. et al. 1958, J. Bacteriol., 76, 15-23.
28. Huhtanen, C.N. 1955, Proc. Soc. Exptl. Biol. Med., 88, 311-312.
29. Prescott, J.M. and Stutts, A.L. 1955, J. Bacteriol., 70, 285-288.
30. Wright, D.E. 1960, J. Gen. Microbiol., 22, 713-725.
31. Huhtanen, C.N. et al. 1954, J. Bacteriol., 68, 749-751.
32. Anderson, R.L. and Ordal, E.J. 1961, J. Bacteriol., 81, 139-146.
33. Allison et al. 1959, Arch. Biochem. Biophys., 84, 245-247, and unpublished data.
34. Wegner, G.H. 1960, U. of Wisconsin, personal communication.
35. Bryant, M.P. et al. 1959, J. Dairy Sci., 42, 1831-1847.
36. Allison M.J. et al. 1958, Sci., 128, 474-475.
37. Bryant, M.P. and Robinson, I.M. 1960, Unpublished data.
38. Hungate, R.E. 1960, Bacteriol. Revs., 24, 353-364.
39. Gall, L.S. et al. 1949, J. Animal Sci., 8, 441-449.
40. Maki, L.R. and Foster, E.M. 1957, J. Dairy Sci., 40, 905-913.
41. Blackburn, H. and Hobson, P. 1960, personal communication.
42. Wegner, G.H. and Foster, E.M., J. Dairy Sci., 43, 566-568.